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Abstract

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Human factor VIII can be packaged and functionally expressed in an adeno-associated virus background: applicability to haemophilia A gene therapy

Dmitri V. Gnatenko, Evgueni L. Saenko, Jolyon Jesty, Liang-Xian Cao, Patrick Hearing & Wadie F. Bahou

Adeno-associated virus (AAV) is a single-stranded DNA parvovirus displaying several attractive features applicable to haemophilia A gene therapy, including non-pathogenicity and potential for long-term transgene expression from either integrated or episomal forms. We have generated and characterized two B-domain-deleted (BDD) fVIII mutants, deleted in residues Phe^{756} to Ile^{1679} (fVIIIΔ756-1679) or Thr^{761} to Asn^{1639} (fVIIIΔ761-1639). [^{35}S]metabolic labelling experiments and immunoprecipitation demonstrated intact BDD-fVIII of the predicted size in both lysates and supernatants (M_r ~ 155 kD for fVIIIΔ756-1679 and M_r ~ 160 kD for fVIIIΔ761-1639) after transient transfection into COS-1 cells. Functional fVIII quantification appeared maximal using fVIIIΔ761-1639, as evaluated by Coatest and clotting assay (98 ± 20 mU/ml/1×10^6 cells and 118 ± 29 mU/ml/1×10^6 respectively, collection period 48 h). To bypass potential size limitations of rAAV/fVIII vectors, we expressed fVIIIΔ761-1639 using a minimal human 243 bp cellular small nuclear RNA (pHU1-1) promoter, and demonstrated fVIII activity ~30% of that seen using CMV promoter. This BDD-fVIII (rAAV(pHU1-1) fVIIIΔ761-1639) can be efficiently encapsidated into rAAV (107% of wild type), as demonstrated by replication centre and DNAase sensitivity assays. A concentrated recombinant viral stock resulted in readily detectable factor VIII expression in COS-1 cells using a maximally-achievable MOI ~35 (Coatest 15 mU/ml; clotting assay 25 ± 2.0 mU/ml/1×10^6 cells). These data provide the first evidence that rAAV is an adaptable virus for fVIII delivery, and given the recent progress using this virus for factor IX delivery *in vivo*, provide a new approach towards definitive treatment of haemophilia A.

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NEW APPROACHES TO GENE TRANSFER/THERAPY

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GENERATION AND CHARACTERIZATION OF RECOMBINANT ADENO-ASSOCIATED VIRAL (rAAV) VECTORS FOR FACTOR VIII GENE THERAPY. D. Gnatenko*, P. Heaton*, J. Genet*, J. Jasht* and W. Bahou. State University of New York, Stony Brook, NY

Coagulation factor VIII (FVIII) contains a domain structure of A1-A2-B-A3-C1-C2, with a large B domain that is dispensable for procoagulant activity. Using oligonucleotide-directed precise gene fusion by PCR (polymerase chain reaction), we have generated and characterized two recombinant B-domain-deleted (BDD) FVIII mutants, specifically deleted in amino acid residues Phe^{745} through Ile^{1670} (FVIIIΔ756-1679), or residues Thr^{760} through Asn^{1639} (FVIIIΔ760-1639). Thrombin cleavage sites Arg^{740} and Arg^{1689} are intact in both mutants, while the previously identified von Willebrand factor (vWF) binding site Glu^{1648} to Arg^{1689} is partially deleted in FVIIIΔ760-1679, although the critical Tyr^{1680} remained intact. To further characterize these mutants, transient transfection assays were completed in COS-1 cells. [^{35}S]-Methionine metabolic labeling experiments and immunoprecipitation using an anti-FVIII light chain monoclonal antibody demonstrated intact BDD FVIII of the predicted size in both lysates and supernatants (Mr ~167 for FVIIIΔ756-1679 and Mr ~161 for FVIIIΔ760-1639). Quantification of functional FVIII expression (by chromogenic assay) in COS-1 cells over a 6-hour collection period demonstrated 10 mU and 60 mU/10^6 cells for FVIIIΔ756-1679 and FVIIIΔ760-1639, respectively. To determine if FVIII could be assembled in an AAV background, we generated rAAV/FVIIIΔ756-1679 driven by a core vWF promoter (342 bp), and containing an SV40 early gene polyadenylation signal. This construct, which is 111% of wild-type AAV, was used for the generation of recombinant viral stocks using cotransfection of 293 cells. Initial replication center assay using 293 viral lysates confirmed that rAAV/FVIIIΔ756-1679 could replicate and package with an estimated titer determination of ~5.5 x 10^4 infectious units (i.u.)/mL. No contaminating wild-type AAV was evident at a sensitivity of 100 i.u./mL. Southern blot analysis of Hirt DNA using a stably-expressing *rep/cap* HeLa cell line demonstrated monomeric and dimeric rAAV forms, with no evidence for contaminating adenovirus. These data establish that a novel BDD functionally active FVIIIΔ756-1679 mutant under a vWF cellular promoter can be successfully packaged in recombinant AAV for the purposes of hemophilia A gene therapy. Given potential advantages for adeno-associated viruses in gene delivery (integration, less pronounced host immunological response), these observations provide an alternative to current adenoviral or retroviral-mediated delivery methods.

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RECOMBINANT ADENO-ASSOCIATED VIRUS MEDIATED GENE TRANSFER INTO HUMAN LEUKEMIA CELL LINES; EFFICIENCIES AND INTEGRATION SITES. T. Itou*, K. Miyamura, A. Abe*, N. Iijima*, N. Emi*, M. Tanimoto*, H. Saito. First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, Japan

Adeno-associated virus (AAV) based vector is one of the promising gene transfer vehicles by virtue of the characteristics of wild-type AAV: tropism to a wide range of human tissues and locus specific integration at chromosome 19q13.3. To elucidate the nature of the recombinant AAV, transduction of neomycin phosphotransferase enzyme gene (NeoR gene) into seven human leukemia cell lines was performed. Transduction efficiencies were assessed by colony formation assay and by the limiting dilution assay. The results from both assays are highly comparable. Transduction efficiencies of the NeoR gene into K-562, MEG-01, Raji, MOLT-3, HL-60, U937 and NKM-1 at an MOI of 1 were 2.7%, 2.6%, 0.16%, 0.09%, 0.09%, <0.025% and <0.025%, respectively. A linear relationship between the transduction efficiency and MOI was observed and this finding implies that higher concentrations of rAAV stock will result in adequate transduction efficiencies. Due to the stability of the virus particles, higher titer rAAV stocks can be made by aggressive concentration methods. Integration of the NeoR gene into host genome was detected by Southern blotting analysis. Various sizes of restriction fragments suggested random integration. Fluorescent in-situ hybridization (FISH) study was carried out in four MEG-01 and two K-562 clones. The integration site in four clones were identifiable and the NeoR gene existed on chromosome 1q or 2q or 13, other than chromosome 19q13.3. Thus rAAV vector without Rep gene can integrate itself stably, but its target site is not localized at one specific position. Further improvements will be necessary for rAAV vector not to lack the feature of site-specific integration without cytotoxicity of Rep.

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REACTIVATION OF SILENCED, VIRALLY TRANSDUCED GENES BY INHIBITORS OF HISTONE DEACETYLASE. WenYong Chen*, Evans Bailey*, Jian-Yun Dong*† and Tim M. Townes. Department of Biochemistry and Molecular Genetics, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL; †Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA.

Retroviral and Adeno-Associated Viral (AAV) sequences can dramatically silence transgene expression in mice. We now report that this repression also occurs in stably infected HeLa cells when the cells are grown without selection. Expression of a transduced lacZ gene (rAAV/lacZ) is silenced in greater than 90% of cells after 60 days in culture. Surprisingly, high-level expression can be reactivated by treating the cells with sodium butyrate or trichostatin A (TSA) but not with 5-azacytidine. When cell clones with integrated copies of rAAV/lacZ were isolated, lacZ expression was silenced in 60% of the clones; however, LacZ expression was reactivated in all of the silenced clones by treatment with butyrate or trichostatin A. TSA is a specific inhibitor of histone deacetylase; therefore, we propose that hyper-acetylation of histones after drug treatment changes the structure of chromatin on integrated viral sequences and relieves repression of transduced genes. The reactivation of silenced, transduced genes has implications for gene therapy. Efficient viral gene transfer followed by drug treatment to relieve suppression may provide a powerful combination for treatment of various genetic and infectious diseases.

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NF-IL6 MODULATES THE TRANSCRIPTIONAL ACTIVITY OF P5 PROMOTOR OF ADENO-ASSOCIATED VIRUS TYPE 2. T. Itou*, H. Iida*, M. Towatari*, S. Tauzuki*, N. Iijima*, M. Tanimoto*, K. Miyamura, H. Saito. First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, Japan

Introduction and Purpose The human adeno-associated virus type 2 (AAV2) requires the coinfection with adenovirus for the optimal replication in host cells, and the precise helper function of adenovirus has not been clarified. There is a binding site for adenovirus E1A in p5 promotor of AAV2 and E1A plays an important role in replication of AAV2. NF-IL6 (C/EBPβ) regulates E1A responsive element however its function for p5 promotor is uncertain. NF-IL6 is also known to be up-regulated under IL-6 stimulation in hepatocyte. The purpose of this study is to clarify the effect of IL-6 for production of rAAV and the transcriptional modulation of p5 promotor with NF-IL6. **Materials and Methods** (1) rAAV Production; pAAV/Ad and pAAV/Neo were cotransfected into adenovirus infected Alexander cells, which derived from human hepatoma, and cells were incubated with or without IL-6 (10u/ml) for 72 hours. Each cell extract was added to HeLa cells and the titer of rAAV was estimated from the number of Neomycin-resistant colony. (2) Promotor Assay of p5; We constructed pGL-p5 that contains the sequence located at -96 to +37 of p5 promotor of AAV2 at the upstream of firefly luciferase reporter gene. pGL-p5 was transfected with or without NF-IL6 expression plasmid, (from Dr. T. Kishimoto) into HeLa or 293 cells, and forty-eight hours later cells were harvested and luciferase assay was performed. **Results** (1) The titers of rAAV/Neo produced with IL-6 and without IL-6 were 250 and <20 colony/plate, respectively. (2) As shown in the table below, in HeLa and 293 cell line, NF-IL6 activated transcription 10-fold and 1-fold, respectively.

NF-IL6	−	+
	Mean (SD)	Mean (SD)
HeLa	1.3×10^5 (2.3×10^5)	1.2×10^7 (1.4×10^7)
293	3.6×10^6 (6.2×10^6)	3.8×10^6 (5.8×10^6)

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Stable transgene expression in hematopoietic cells transduced with bicistronic retrovirus vectors containing GFP selectable marker gene. A. Kume, K. Matsuda, Y. Ueda, M. Urabe, T. Suda and K. Ozawa. *Department of Molecular Biology, Institute of Hematology, Duchy Medical School, Tochigi; CREST, JST, Saitama; and Department of Cell Differentiation, Institute of Molecular Genetics and Embryology, Kumamoto University School of Medicine, Kumamoto, Japan.*

Recombinant retrovirus vectors are most widely employed in gene therapy trials targeting hematopoietic cells. However, the transduction efficiency via retroviruses is still insufficient, particularly for hematopoietic stem cells. This is a major drawback in applying retrovirus vectors to larger animals and evaluation of the transgene expression *in vivo* has been hampered. One way to overcome the efficiency problem is to develop a system to enrich the transduced hematopoietic stem cells without losing their totipotency. For this purpose, we studied the feasibility of green fluorescent protein (GFP) gene as a rapid selectable marker of retrovirally transduced cells. This marker would also facilitate the identification and tracking of the progeny of transduced stem cells *in vivo*. We constructed several bicistronic retroviruses, one of which was designed to express EGFP (Clontech) under control of the encephalomyocarditis virus (EMCV)-derived internal ribosome entry site (IRES), while the human CD24 gene was placed to be expressed in cap-dependent manner (MSCV/CD24-IRES-EGFP). We transduced several cell lines and the primary murine bone marrow cells and observed co-expression of CD24 and GFP in those cells. The efficient transgene expression in Ba/F3 pro-B cells has been sustained for more than 6 months. In the marrow-reconstituted mice, GFP expression was detected in 25-40% of the donor-derived peripheral blood cells on day 45 post-transplantation and further long-term expression *in vivo* is currently under investigation. These results indicate the GFP-tagged bicistronic retrovirus vectors are suitable for marking hematopoietic stem cells and thus would work as valuable tools to track the transduced cells in recipients.

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Retroviral mediated transfer and expression of the human glucose 6-phosphate dehydrogenase (G6PD) gene in mouse bone marrow cells. A. Rovira, H. Gallardo, M. De Angioletti, C. Murphy, V. Rosti, D. Liu, M. Sadelain and L. Luzzatto. *Department of Human Genetics, MSKCC, New York, NY.*

The clinical manifestations of G6PD deficiency are mostly mild or limited to acute episodes. However, a small subset of G6PD deficient subjects have a severe chronic non-spherocytic hemolytic anemia (CNSHA). Since G6PD inheritance is X-linked, the heterozygous mothers of these male patients are genetic mosaics as a result of X-chromosome inactivation and their blood is often normal, suggesting somatic cell selection in favor of the hematopoietic cells with the normal G6PD allele on the active X-chromosome. Based on this observation, and since no satisfactory treatment for CNSHA is available, we have constructed two sets of murine leukemia-based retroviral vectors in which expression of the human G6PD (hG6PD) cDNA is driven either by the retroviral LTR of Myeloproliferative Sarcoma Virus (MPSV) or by the G6PD promoter itself. In the latter vector, the G6PD intron 12 and the β-globin polyadenylation signal were cloned in reverse orientation. To generate the vectors, each of the plasmid constructs was transfected into the ecotropic ΨCRE packaging cell line and stable producers were selected. To assess the ability of each vector to transfer and express the hG6PD cDNA we first used NIH/3T3 mouse fibroblast. To measure the expression of hG6PD in transduced cells we combined spectrophotometric quantitation of G6PD activity and cellulose acetate gel electrophoresis, which resolves human (retrovirally transferred) from mouse (endogenous) G6PD activity. We found that LTR and G6PD promoter driven vectors both stably integrate and produce enzymatically active hG6PD. From the analysis of individual transduced fibroblast cell clones we determined that the level of expression was roughly proportional to the copy number of integrated provirus as assessed by Southern blotting. The hG6PD activity from integration of a single copy of the hG6PD gene was on average comparable to mouse G6PD activity. We next used the gpg29 packaging cell line for the production of virions pseudotyped with the G glycoprotein of vesicular stomatitis virus (VSV-G). This enabled us to obtain high titer supernatants (3×10^7 infectious particles/ml) which were used for G6PD transduction into mouse bone marrow cells: this achieved expression of hG6PD up to 10 times the level of mouse G6PD in short-term bone marrow cultures. We are currently testing whether, upon transplantation into syngeneic lethally irradiated mice, transduced hematopoietic cells will be capable of long-term expression and possible self-selection *in vivo*, comparing the promoter of the housekeeping gene G6PD to that of MPSV.

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Recombinant adeno-associated virus as a vehicle for gene delivery of human mutant factor VIII. D. Gnatenko, J. Jesty, P. Hearing, E. Ssenko and W.F. Bahou. *State University of New York at Stony Brook, NY, and American Red Cross, Rockville, MD.*

Adeno-associated virus (AAV) is a single-stranded DNA parvovirus displaying several attractive features applicable to hemophilia A gene therapy, including non-pathogenicity and potential for long-term transgene expression from either integrated or episomal forms. Size limits of encapsulation (~4.6 kb) restrict the use of this vector for delivery of the full-length human FVIII cDNA. Using oligonucleotide-directed precise gene fusion by PCR, we have generated and characterized two recombinant B-domain deleted FVIII (BDD-FVIII) mutants, specifically deleted in amino acid residues Phe^{746} through Ile^{1649} (FVIIIΔ756-1679), or residues Thr^{761} through Asn^{1629} (FVIIIΔ760-1639). [^{35}S] metabolic labeling experiments and immunoprecipitation using the anti-FVIII light chain monoclonal antibody ESH4 demonstrated intact BDD-FVIII of the predicted size in both lysates and supernatants (Mr ~ 155 kDa for FVIIIΔ756-1679 and Mr ~ 160 kDa for FVIIIΔ760-1639) after transient transfection into COS-1 cells. Functional FVIII quantification appeared maximal using FVIIIΔ760-1639, as evaluated by both Coatest and clotting assay determination (294 ± 60 mU/ml/1 x 10^6 cells and 354 ± 87 mU/ml/1 x 10^6 respectively, collection period 48 hours). The diminished activity of FVIIIΔ756-1679 presumably reflects instability related to deletion of interactive residues mediating von Willebrand factor (vWF) binding. To determine whether BDD-FVIII can be encapsulated into rAAV we have generated rAAV/FVIIIΔ756-1679 driven by a minimal vWF core promoter (342 bp) also containing the SV40 early gene polyadenylation signal. This construct, which is 111% of wild-type AAV, demonstrated a titer 5×10^4 infection units (i.u.)/ml., as evaluated by replication center assay on 293 cells. Purification by CsCl gradient centrifugation generated a concentrated stock of 1×10^7 i.u./ml, with no evidence for contaminating wild-type AAV. Southern blot analysis of Hirt DNA using a stable-expressing *rep/cap* cell line demonstrated replicative monomeric and dimeric forms of rAAV, with no evidence for contaminating adenovirus. Infection of COS-1 cells with rAAV/FVIIIΔ756-1679 (multiplicity of infection (MOI) – 10 i.u./cell for 24 hours) resulted in secretion of detectable amounts of functionally active BDD-FVIII (Coatest - 63 ± 9 mU/ml/1 x 10^6 cells; clotting assay - 60 ± 12 mU/ml, ELISA - 15.9 ± 1.8 ng/ml for 48 hours). Higher yield of functionally active BDD-FVIII may be achieved using higher MOI, more efficient promoter, or BDD-FVIII with intact vWF binding site. Taken together, these data provide the first evidence that AAV-based vectors can be successfully used for packaging of BDD-FVIII cDNA into an AAV background with secretion of a functionally active protein even at low MOI.

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Therapeutic levels of human protein C in rats after retroviral vector-mediated hepatic gene therapy. S.-R. Cai, S.C. Kennedy, W.M. Bowling, M.W. Flye and K.P. Ponder. *Dept. of Internal Medicine, Washington University School of Medicine, St. Louis, MO.*

Homozygous protein C deficiency results in a serious thrombotic disorder that might be treated by expressing a normal human protein C (hPC) gene in patients. An amphotropic retroviral vector with a strong liver-specific promotor and the hPC cDNA was delivered to rat hepatocytes *in vivo* during liver regeneration. Expression of hPC in 7 rats varied from 55 to 203 ng/ml (1.3-5% of normal) for 2 weeks after transduction. Expression increased 4- to 9-fold to an average level of 900 ng/ml (22% of normal) in four rats at ~1-2 months and remained stable thereafter for 1 year. These rats all developed high titer anti-hPC antibodies and exhibited a prolonged hPC halflife *in vivo*. Expression was stable at 160 ng/ml (4% of normal) for 1 year in one rat, who did not develop antibodies against hPC. Expression fell to <50% of the initial levels at 1-2 months after transduction in the 2 remaining rats, both of whom developed high-titer anti-hPC antibodies. The hPC functional activity was tested. One assay used a human specific antibody to immuno-precipitate hPC, which was then activated with Protac and incubated with a chromogenic substrate. A second assay involved activation of the hPC in rat plasma with Protac followed by testing its ability to inhibit the clotting time in an APTT assay using hPC-deficient human plasma. In all cases, the functional hPC activity was similar to or higher than the antigen levels. We conclude that most transduced rats achieved hPC levels that would prevent purpura fulminans in humans. Hepatic gene therapy might therefore become a viable treatment for patients with severe homozygous hPC deficiency. We also conclude that anti-hPC antibodies increased the hPC half-life and plasma levels in some rats, but did not interfere with its functional activity. Some investigators have presumed that the development of antibodies during a gene therapy protocol will increase the clearance of a protein. This study demonstrates that the antibodies directed against a plasma protein do not necessarily lead to increased clearance or abrogation of a biological effect.

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Re-evaluation of the *ex vivo* autologous fibroblast transduction model in rabbits: Achievement of long term (>600 days) factor IX expression in a small percentage of animals. Lin Chen, David Nelson, Zhili Zheng and Richard Morgan (Intr. by J.N. Lozier). *Clinical Gene Therapy Branch, National Human Genome Research Institute, NIH, Bethesda MD 20892.*

Hemophilia B is caused by mutations in factor IX and is a well studied model for gene therapy. Based *in vitro* data, primary fibroblasts have been shown to express high levels of factor IX following transduction by retroviral vectors. *Ex vivo* gene therapy using retroviral transduced primary fibroblasts has been investigated by many researchers. Published results on the effectiveness of this approach are inconsistent and some are even in conflict. We felt it was important to reevaluate the *ex vivo* approach using newly designed retroviral vectors in a large cohort of rabbits. In this study, we first measured the kinetics of human factor IX in rabbits, including half life, volume of distribution and bioavailibility with intravenous, intraperitoneal or subcutaneously administration. We then tested a transplantation protocol that uses a simple subcutaneous injection of a mixture of retroviral transduced primary fibroblasts in a collagen suspension. 15 rabbits were subject to this procedure and two of them showed long term (>600 days) expression of human factor IX in plasma. Histological examination of the injection sites showed an increase of vascularity but no other pathological changes. No significant difference between animals with detectable factor IX expression and those without were documented at injection sites. PCR and RT-PCR studies show the existence of the implanted cells and variable degrees of expression of transgene at the injection site in all animals. In addition, we analyzed the antibody response to different components in implantation mixture as well as to the transgene products including the human factor IX and neomycin phosphotransferase (Neo). Overall, our result suggests that the rabbit can be a useful model for *ex vivo* gene therapy for hemophilia B. *Ex vivo* gene delivery using primary autologous fibroblasts has great potential, especially with improved retroviral vectors. The key to successful *ex vivo* gene therapy will depend on significant improvements over the currently used implantation methodology.